The Presence of *Agrobacterium tumefaciens* Plasmid DNA in Crown Gall Tumour Cells

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SUMMARY

DNA hybridization studies indicate the presence of DNA complementary to *Agrobacterium tumefaciens* plasmid in bacterium-free crown gall tumour cells. The amount of this DNA is estimated to be about 0.1 % of the tumour cell DNA.

INTRODUCTION

Extracellular infections of wounded dicotyledonous plants by the Gram-negative bacterium, *Agrobacterium tumefaciens* (Smith and Townsend, Conn.) result in the transformation of normal plant cells to tumour cells. These crown gall tumour cells will continue to grow autonomously in the absence of the bacterium (Braun, 1943). Various mechanisms have been proposed for the induction of these tumours, including the transfer of genetic information (either as DNA or as RNA) from the bacterium to the plant cell (Srivastava, 1965; Braun & Wood, 1966; Schilperoort *et al.* 1967; Guille, Quetier & Huguet, 1968; Milo & Srivastava, 1969; Quetier, Huguet & Guille, 1969; Schilperoort, van Sittert & Schell, 1973; Zaenen *et al.* 1974).

Direct evidence from nucleic acid hybridization studies for the presence of DNA complementary to bacterial DNA in bacterium-free crown gall tumour cells has been presented by some authors (Srivastava, 1965; Schilperoort *et al.* 1967; Milo & Srivastava, 1969; Quetier *et al.* 1969). The estimated amount of DNA involved seems very large, between 1 and 9 bacterial genome-equivalents per plant cell (Quetier *et al.* 1969; Schilperoort *et al.* 1973). However, there are also hybridization studies indicating the absence of such large amounts of DNA complementary to bacterial DNA in crown gall tumour cells (Chilton *et al.* 1974*a, b*; Eden *et al.* 1974; Drlica & Kado, 1974).

The presence of plasmid DNA in *A. tumefaciens* and its correlation with the virulence of various strains (Van Larebeke *et al.* 1974; Zaenen *et al.* 1974) suggested that it would be of interest to examine the possible role of bacterial and plasmid DNA in tumour induction by using DNA:DNA hybridization studies.

METHODS

Media. Bacteria were grown in minimal salts medium containing (g/l): NH_4Cl , 5; NH_4NO_3 , 1; Na_2SO_4 , 2; K_2HPO_4 , 3; KH_2PO_4 , 1; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1, with 0.2 % glucose and 50 μCi methyl [^3H]thymidine or 10 μCi $^{32}\text{P}_4/\text{ml}$ (Clowes & Hayes, 1968). Plant tissue-

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culture cells of normal *Vinca rosea* were grown in White's medium (Wood & Braun, 1961) supplemented with (mg/l): α -naphthalene acetic acid, 1; kinetin, 0.5; asparagine, 200; glutamine, 200; *m*-inositol, 100; AMP, 100; GMP, 100. *Vinca rosea* crown gall tumour cells were grown on unsupplemented White's medium. All cultures were grown at 25 °C, with 14 h of light/day.

Organisms. *Agrobacterium tumefaciens* strain A6, normal *Vinca rosea* tissue-culture cells (NV), and bacterium-free *Vinca rosea* crown gall tissue culture strains v_{A6} and v_{BP} were obtained from Professor A. Braun.

Isolation of DNA. Plant-cell DNA was extracted by the method of Kirby (1957). Cells from cultures which had not yet reached the stationary phase of growth were frozen on dry ice and ground in 0.3 M-sodium trichloroacetate pH 6.5 in a Waring blender. The homogenate was filtered through two layers of cheesecloth and extracted with an equal volume of phenol. The aqueous layer was then mixed with an equal volume of 2-ethoxyethanol; the precipitate was redissolved in 0.3 M-sodium trichloroacetate pH 6.5 and reprecipitated three times. The precipitate was then dissolved in H₂O, and 4 g sodium acetate/100 ml were added and dissolved, followed by an equal volume of 2-ethoxyethanol. The precipitate was then dissolved in 0.1 $\times$ SSC (saline sodium citrate, where 1 $\times$ SSC contains 0.15 M-NaCl and 0.015 M-trisodium citrate) and digested for 90 min at 37 °C with RNAase A (Sigma; 20 μ g/ml, heated at 100 °C for 10 min before use) followed by pronase (Calbiochem; 200 μ g/ml, self-digested for 90 min before use) for 120 min. The DNA was then purified by extraction with chloroform containing 100 ml *n*-octanol/l. The extraction was repeated at least four times, and the DNA precipitated with 2 vol. of cold ethanol. The precipitate was redissolved in 0.1 $\times$ SSC. Purified DNA preparations in 0.1 $\times$ SSC had E_{260}/E_{280} ratios between 1.70 and 1.85. No RNA was detectable by examination of the supernatant from the base hydrolysis (0.3 M-KOH, 18 h, 37 °C) of 40 μ g DNA/ml for E_{260} .

Bacteria were lysed by using the freeze-thaw lysozyme technique (Kohler, Ron & Davis, 1966) followed by the addition of 0.1 % sodium dodecyl sulphate. The lysate was extracted with chloroform-isoamyl alcohol (24:1, v/v) four times (Marmur, 1961). The aqueous layer was added to 2 vol. of cold ethanol and the DNA wound out of solution. The DNA was dissolved in 0.1 $\times$ SSC and digested for 90 min at 37 °C with RNAase A (Sigma; 20 μ g/ml, heated at 100 °C for 10 min before use) followed by pronase (Calbiochem; 200 μ g/ml, self-digested for 90 min before use) for 120 min. The solution was extracted three times with chloroform and added to 2 vol. of cold ethanol. The DNA was wound out of solution and dissolved in 0.1 $\times$ SSC. Purified DNA preparations in 0.1 $\times$ SSC had E_{260}/E_{280} ratios between 1.75 and 1.85. No RNA was detectable by examination of the supernatant from the base hydrolysis (0.3 M-KOH, 18 h, 37 °C) of 400 μ g DNA/ml for radioactivity or E_{260} .

Bacterial-plasmid DNA was prepared from the supernatant of the salt precipitation of chromosomal DNA from bacteria lysed by using sodium dodecyl sulphate and lysozyme (Guerry, Le Blanc & Falkow, 1973). The supernatant was diluted with an equal volume of 0.01 M-tris-HCl-buffer pH 8.0 and layered on a linear 5 to 20 % sucrose gradient and centrifuged at 300 000 g in an SW65 rotor for 1 h. Fractions were collected and those containing the plasmid peak (as determined by radioactivity) were pooled and dialysed against 0.1 $\times$ SSC. Only a single band of radioactivity with an estimated molecular weight of 10^8 was observed on these sucrose gradients. This band of plasmid DNA would include open-circular plasmid DNA and linear plasmid DNA, as well as any covalently-closed-circular plasmid DNA which was relaxed by the sodium dodecyl sulphate treatment. The plasmid DNA preparation was then digested with RNAase and pronase, and chloroform-extracted and ethanol-precipitated as described above for plant DNA. ¹²⁵I-labelled DNA was prepared

by using a modification of the technique of Prensky, Steffenson & Hughes (1973) and purified by chromatography on hydroxyapatite. Radioactive DNA to be used in the liquid phase of filter-paper hybridization reactions and the plant and plasmid DNAs used in hybridization reactions in solution, were degraded to a molecular weight of approximately 2×10^5 by a limited depurination reaction (McConaughy & McCarthy, 1967). The DNA was dissolved in 0.1 M-sodium acetate pH 4.2 and heated at 70 °C for 33 min. The pH of the solution was adjusted to 12 with NaOH and the solution heated at 50 °C for 10 min. It was then dialysed against $1.5 \times \text{SSC}$.

^3H -labelled adenovirus SA7 DNA was kindly provided by Dr J. P. Burnett. It was extracted and labelled by following the procedures of Burnett & Harrington (1968) and Burnett *et al.* (1968). Radioactive bacterial DNA was prepared by growing the bacteria in minimal salts medium with 25 to 50 μCi methyl [^3H]thymidine/ml or 10 μCi $^{32}\text{PO}_4$ /ml. The specific activities obtained ranged from 5×10^3 to 7×10^4 c.p.m./ μg for ^3H -DNA and from 1×10^3 to 8×10^3 c.p.m./ μg for $^{32}\text{PO}_4$ -DNA.

DNA hybridization reactions. The filter-paper DNA hybridization technique of Denhardt (1966) was used. Double-stranded DNA was denatured by exposure to high pH. It was then neutralized to pH 7 and trapped on Millipore GS filter papers by filtration in $6 \times \text{SSC}$, dried, and heated at 80 °C under a vacuum for 2 h. The filter papers were placed in pre-incubation medium [0.02 % (w/v) bovine serum albumin (Armour; fraction V), 0.02 % (w/v) Ficoll (Pharmacia; average mol. wt 400000) and 0.02 % (w/v) polyvinylpyrrolidone (Sigma; average mol. wt 360000)] in $1.5 \times \text{SSC}$ at 67 °C for 6 to 12 h and then reacted with radioactive DNA in 3 ml $1.5 \times \text{SSC}$ at 67 °C for 18 or more hours. The filter papers were removed, washed in $1 \times \text{SSC}$, dried, and counted in a liquid scintillation counter. The melting temperature of the radioactive DNA hybrids was determined by removing the filter paper from the toluene scintillation fluid, washing it with toluene, drying it, placing it in $0.1 \times \text{SSC}$ at the desired temperature for 30 to 60 min, washing it with $0.1 \times \text{SSC}$, drying the filter paper, and recounting it.

Hybridization reactions in solution were carried out in $1.5 \times \text{SSC}$ at 67 °C. The DNA was denatured by exposure to high pH. The DNA concentration in the reactions was kept constant at 150 $\mu\text{g}/\text{ml}$ by adding calf-thymus DNA (Sigma; type V) or normal *V. rosea* DNA, both of which were degraded to a molecular weight of 2×10^5 by using the limited depurination reaction described above. Total radioactive DNA was measured by trichloroacetic acid (TCA) precipitation using bovine serum albumin as a carrier, followed by filtration on GF/A filters which were washed with ethanol and ether, dried, and counted in a liquid scintillation counter in toluene containing 5 g PPO and 0.3 g POPOP/l. Double-stranded radioactive DNA was measured by incubating the sample (10 μg or less) with 2 units of *Neurospora crassa* endonuclease (Miles Laboratories) in $1.5 \times \text{SSC}$ at 37 °C for 30 min to degrade single-stranded DNA. The remaining double-stranded DNA was precipitated by TCA, collected by filtration on GF/A filters, and counted.

RESULTS

Filter-paper hybridization reactions

The results of filter-paper hybridization reactions are shown in Table 1. The amount of bacterial DNA which hybridized with tumour-cell DNA was variable, depending on the preparation of bacterial DNA used. The range of values for bacterial DNA hybridized to 40 μg of tumour-cell DNA was 0.5 to 15 ng for all bacterial DNA preparations, and 2 to 15 ng for bacterial DNA from stationary-phase cultures. Each individual DNA preparation

Fig. 1

Fig. 1. Melting curves of hybridization reactions. The melting was carried out in $0.1 \times \text{SSC}$. $\circ$, *Agrobacterium tumefaciens*:*A. tumefaciens* hybrid; $\bullet$, *A. tumefaciens*:*V. rosea* tumour v_{A6} hybrid; $\blacktriangle$, *A. tumefaciens* plasmid DNA:*V. rosea* tumour v_{A6} hybrid.

Fig. 2

Fig. 2. Hybridization of *V. rosea* v_{A6} tumour DNA with increasing amounts of *A. tumefaciens* A6 plasmid DNA. The filter papers contained $40 \mu\text{g}$ of v_{A6} DNA. The hybridization reaction was at 67°C in $1.5 \times \text{SSC}$.

Table 1. Filter-paper hybridizations

Radioactive DNA in solution	Radioactive DNA (ng) bound to filter papers containing $40 \mu\text{g}$ DNA from		
	Exponential-phase <i>A. tumefaciens</i> A6	Normal <i>V. rosea</i>	Tumour <i>V. rosea</i> v_{A6}
Stationary-phase <i>A. tumefaciens</i> A6 ($2 \mu\text{g}$)*	327	0.7	4.2
Exponential-phase <i>A. tumefaciens</i> A6 ($2 \mu\text{g}$)*	282	0.8	0.9
Plasmid from <i>A. tumefaciens</i> A6 ($0.02 \mu\text{g}$)†	8.6	0.0	15.7

* Numbers are averages of at least ten reactions.

† The amount of plasmid DNA was calculated assuming the specific activity of plasmid DNA was the same as that of chromosomal DNA. Numbers are averages of five reactions.

gave consistent ($\pm 15\%$) results on duplicate experiments. We believe this variation to be due to variations in the amount of plasmid DNA contained in the bacterial DNA preparation since the percentage of plasmid DNA varies with the growth phase. In minimal salts medium the percentage of plasmid DNA is highest in stationary-phase cultures (Matthysse, unpublished observations). The hybridization of bacterial DNA to tumour-cell DNA was also highest with bacterial DNA from stationary-phase cultures. The hybridization of bacterial DNA with normal plant-cell DNA was too small to be significant with this technique, and all of this hybrid melted at a temperature below 55°C in $0.1 \times \text{SSC}$. Similar data were obtained when ^{125}I -labelled bacterial DNA was used in the hybridization reaction. The melting curves of such filter-paper hybrids between radioactive bacterial DNA and non-radioactive bacterial DNA or tumour-cell DNA are shown in Fig. 1. Eighty-five % of each hybrid melted at a temperature above 70°C .

To determine whether plasmid DNA was indeed involved in this hybridization reaction between bacterial and tumour-cell DNA, purified plasmid DNA was prepared and used in the hybridization reaction. Although no plasmid DNA could be seen to hybridize to normal-cell DNA, the plasmid DNA did hybridize to about 0.04% of tumour-cell DNA (Table 1). The melting profile of this hybrid indicated that 80% of the hybrid was stable at 70°C and melted between 75 and 85°C (Fig. 1).

Increasing amounts of plasmid DNA were added to the hybridization reaction to obtain a saturation value for the amount of tumour-cell DNA complementary to plasmid DNA (Fig. 2). Approximately 0.1 % of the tumour-cell DNA appeared to be complementary to bacterial-plasmid DNA.

Liquid hybridization reactions. To obtain another estimate of the amount of DNA complementary to bacterial DNA present in a tumour cell, hybridization reactions in solution were carried out using radioactive bacterial-plasmid DNA. Such DNA hybridization reactions are bimolecular reactions in which the concentrations of the two reacting complementary strands are equal. The extent of renaturation at any time t can be expressed as

$$\frac{Co}{C} = \frac{K_2 Cot}{2} + 1,$$

where Co is the total concentration of the renaturing sequences, C the concentration of single-stranded sequences, and K_2 the second-order rate constant (Wetmur & Davidson, 1968). If the original renaturing sequences are radioactive and an additional amount of non-radioactive DNA of the same sequences is added to the reaction mixture, then the rate of renaturation of the radioactive sequences will be increased, so that

$$\frac{Co}{C} = \frac{K_2 Co(1+r)t}{2} + 1,$$

where Co and C refer to the concentrations of total and single-stranded radioactive sequences respectively and r is the ratio of non-radioactive sequences to the radioactive sequences (Sharp, Petersson & Sambrook, 1974). If Co/C is plotted against t , in the presence and absence of the additional complementary DNA sequences, then the slope of the lines will be $K_2 Co/2$ and $K_2 Co(1+r)/2$, respectively, and the ratio of the slopes will be $(1+r)$. Thus the amount of tumour-cell DNA which is complementary to bacterial-plasmid DNA can be determined by measuring the rate of renaturation of a known concentration of radioactive bacterial-plasmid DNA in the absence and presence of a known concentration of tumour-cell DNA.

To check this technique for determining the presence of small amounts of non-radioactive plasmid-DNA sequences, the renaturation of radioactive plasmid DNA was measured in the absence and presence of known amounts of non-radioactive plasmid DNA (Fig. 3). The addition of 1.1 μ g non-radioactive plasmid DNA/ml to 1.1 μ g radioactive plasmid DNA/ml increases the slope of the plot of Co/C versus t by 1.95. The predicted increase in slope would be 2.0. Thus the technique seems to be sensitive enough to be used to detect the presence of small amounts of plasmid-DNA sequences in tumour-cell DNA.

The results of such experiments are shown in Fig. 4. ^3H -labelled adenovirus SA7 DNA was included in the reaction mixtures as a control for non-specific increases in the rate of hybridization. The presence of tumour-cell DNA did not affect the rate of renaturation of the adenovirus DNA but it did increase the rate of renaturation of plasmid DNA. The increase in the slope of the line for plasmid DNA renaturation in the presence of tumour-cell DNA indicates that 0.2 % of the tumour-cell DNA was complementary to plasmid DNA.

It is difficult to convert this number into an amount of DNA complementary to plasmid DNA per diploid plant-cell genome as many of the tissue culture cells are aneuploid (Matthysse, unpublished observations).

The Cot_1 for the adenovirus SA7 DNA under these conditions was 0.06. The complexity of the SA7 DNA is 2.2×10^7 (Burnett & Harrington, 1968). Its expected Cot_1 (measured by

Fig. 3. Renaturation of *A. tumefaciens* A6 plasmid DNA. □, Renaturation of 0.44 µg radioactive plasmid DNA/ml, alone; ▲, renaturation of 1.1 µg radioactive plasmid DNA/ml, alone; ○, renaturation of 1.1 µg radioactive plasmid DNA and 1.1 µg non-radioactive plasmid DNA/ml; ●, renaturation of 1.1 µg radioactive plasmid DNA and 3.3 µg non-radioactive plasmid DNA/ml.

Fig. 4. Renaturation of *A. tumefaciens* A6 plasmid DNA and adenovirus SA7 DNA, with and without *V. rosea* V_{A6} tumour DNA. ○, ●, Renaturation of (a) plasmid DNA, and (b) SA7 DNA, in mixture 1; ▲, renaturation of (a) plasmid DNA, and (b) SA7 DNA, in mixture 2. Mixture 1 contained (µg/ml), in 1.5 × SSC: ³²P-labelled *A. tumefaciens* A6 plasmid DNA, 0.8; normal *V. rosea* (●) or calf thymus (○) DNA, 149; ³H-labelled adenovirus SA7 DNA, 0.043. Mixture 2 contained (µg/ml), in 1.5 × SSC: ³²P-labelled *A. tumefaciens* A6 plasmid DNA, 0.8; tumour *V. rosea* V_{A6} DNA, 149; ³H-labelled adenovirus SA7 DNA, 0.043.

extinction which gives a larger Cot_t value than does hydroxyapatite) would be 0.05 (Britten & Kohne, 1965). [Extinction measurements of Cot_t values are probably closer to the values obtained by single-stranded nuclease degradation, which is the procedure used here, than to hydroxyapatite Cot_t measurements, since hydroxyapatite measures unpaired single-stranded tails of double-stranded hybrids as double-stranded (Britten & Kohne, 1965).] The Cot_t for the plasmid DNA was 0.4. If the complexity of the plasmid DNA is 1.1×10^8 (Zaenen *et al.* 1974), its expected Cot_t would be 0.4 (Britten & Kohne, 1965).

DISCUSSION

The evidence presented above supports the hypothesis that *A. tumefaciens* induces tumours by transferring nucleic acid from the bacterium to the plant cell. The nucleic acid involved is apparently the bacterial-plasmid DNA. Earlier difficulties in reproducing results from various laboratories may have been due to variations in the amount of plasmid DNA contained in bacterial, and possibly plant, DNA preparations. The amount of DNA in a plant tumour cell complementary to plasmid DNA is estimated to be between 0.1 and

0.2 % of the plant-cell DNA. The role of this plasmid DNA in transforming a normal cell to a tumour cell remains unknown.

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